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Oxygen transfer strategy modulates the productions of lipase and esterase enzymes by *Candida rugosa*

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ABSTRACT

Different oxygen transfer strategies were employed in a batch bioreactor system to compare the activities, productivities and yields of lipase and esterase enzymes by *Candida rugosa* as well as the biomass accumulation. The fermentation starting with full oxygen saturation and continuing with the level of 30% saturation provided the highest lipase activity whereas continuing with the level of 60% provided the highest esterase activity. Low biomass yield was obtained when the fermentation was conducted at the level of full oxygen saturation. Time courses of the extracellular and intracellular enzyme activities indicated that lipase activity was growth-associated and the cells secreted esterase into the medium after a considerable level of extracellular lipase activity was reached at all oxygen transfer strategies considered.

1. Introduction

Oxygen transfer condition is among the crucial factors that affect the product distribution in fermentation processes. The shortage of oxygen in the fermentation broth can restrict or enhance a metabolite formation by changing metabolic pathways. In some cases, controlled oxygen transfer rates can be required to regulate the oxygen uptake rate by the cells.

Candida rugosa is among the most extensively studied microorganisms by biotechnologists with respect to its powerful lipase (E.C. 3.1.1.3) production capacity. Wide substrate specificity provides *C. rugosa* lipase (CRL) to be successfully used in a variety of hydrolysis and esterification reactions, and its high stereoselectivity and regioselectivity make possible the synthesis of several pharmaceuticals [1]. *C. rugosa* synthesizes and secretes a mixture of lipase isoenzymes differing in biocatalytic properties. It has been established that at least seven genes are involved in the *C. rugosa* lipase-producing machinery and some isoenzymes exhibit esterase (E.C. 3.1.1.1) activity [2]. Additionally, carboxyl-thioesterases were also described and characterized from commercial samples [3,4]. Since *C. rugosa* has a non-universal genetic code [5], difficulties have been arisen in obtaining recombinant CRL to produce the desired isoenzyme. Consequently, many research activities have been diverted into modulating lipolytic enzyme activities towards desired enzyme through changing culture and fermentation conditions or operation mode of bioreactors. As Dominguez de Maria et al. [2] reviewed, carbon source (inducer), kind of operation (batch/fed-batch) and feed rate are important parameters in *C. rugosa* fermentation in terms of quantity and quality of the crude lipase. Despite comprehensive studies on CRL production, those investigate the effects of dissolved oxygen (DO) concentration on the fermentation progress are limited, and, moreover, none of them distinguishes the conditions that enhance lipase and esterase activities individually.

The overview of the literature on the effect of aeration in *C. rugosa* fermentation reveals that DO concentration greatly influences the production of lipase enzyme. It was reported that DO concentration above 20% of the medium saturation was enough to ensure the production of CRL [6]. Sokolovska et al. [7] proposed to use air enriched by pure oxygen in the fermentation of *C. cylindracea*, and reported the flow rates of the gases to maintain the oxygen concentration at the recommended value (20%) for optimum lipase production. Puthli et al. [8] changed the oxygen supply to the bioreactor by changing aeration rate, and observed that higher oxygen concentration beyond the optimum value was detrimental and decreased the biomass production, especially in the late logarithmic phase of growth cycle which in turn affected the overall lipase production. Although oxygen supply conditions are expected to affect not only lipase by also esterase enzyme production by *C. rugosa*, none of the authors have reported the DO concentration or oxygen transfer condition for increased esterase activity.

The aim of the present work is to propose different oxygen transfer strategies (OTSs) that increase lipase and esterase enzyme productions individually in *C. rugosa* fermentation. For all OTSs employed, time variations in extracellular and intracellular lipase

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and esterase activities as well as cell biomass were followed. The overall results indicated that oxygen transfer strategy (OTS) in a bioreactor system was among decisive parameters that affected the activity, productivity and yield of *C. rugosa* enzymes.

2. Materials and methods

2.1. Chemicals

The chemicals were of reagent grade and purchased from commercial suppliers (Merck, Germany; Sigma–Aldrich, USA).

2.2. Microorganism, culture media and conditions

C. rugosa DSMZ 2031 obtained from Deutsche Sammlung von Mikrooganismen and Zellkulturen GmbH (DSM, Braunschweig, Germany) was used in the study. The stock cultures were maintained on universal yeast medium (UYM).

The microorganism incubated at 30 °C for 22 h was inoculated into 100 mL pre-culture medium of the following composition (per liter): 5 g soy peptone, 3 g yeast extract, 3 g malt extract, 10 g dextrose. After incubation at 30 °C and 150 rpm conditions for 22 h in an orbital shaker (Edmund Bühler SM-30, Germany), the cells were transferred to the bioreactor that contained the enzyme production medium (per liter): 2 g triolein, 4 g urea, 15 g KH_2PO_4, 5.5 g K_2HPO_4, 1 g $MgSO_4{\cdot}7H_2O$, 10 mg $FeCl_3{\cdot}6H_2O$ and 10 ml vitamin solution (pH 6.2). The vitamin solution contained per liter 400 mg of thiamine, 2 mg of biotin and 2 g of inositol.

Batch bioreactor experiments were conducted in 2 L bioreactor systems (Sartorius Biostat B Plus, Germany), consisting of temperature, pH, foam, agitation rate, gas flow rate and DO concentration measurements and controls with 1 L working volume. Enzyme production employing different OTSs was carried out at $T = 30$ °C temperature, $N = 500$ rpm agitation rate and Q/V = 0.6 vvm gas flow rate conditions. The pH of the medium was not controlled throughout the fermentation. The changes in extracellular and intracellular activities of lipase and esterase enzymes as well as cell biomass concentration with time were followed. Extracellular and intracellular protease activities were also measured at the end of fermentations.

2.3. Enzyme assays

Alkalimetric final titration was used to determine the lipase enzyme activity by adapting the method by Cernia et al. [9]. The mixture, containing 2.5 mL phosphate buffer solution (0.1 M pH 7.2), 0.5 mL olive oil and 0.1 mL sample, was incubated at 37 °C under magnetic stirring for 30 min. After terminating the reaction with 2.5 mL acetone:ethanol mixture 1:1 (v/v), the solution was titrated with 0.1 M NaOH in the presence of phenolphthalein as indicator. One unit lipase activity (U) was defined as the amount of enzyme that catalyzes the release of fatty acid per min under the conditions mentioned above.

The esterase enzyme activity was measured spectrophotometrically (Shimadzu 1601, Tokyo, Japan) by using *p*-nitrophenyl acetate as substrate [10]. One unit enzyme activity (U) was defined as the amount of enzyme produces 1 μmol *p*-nitrophenol per min at pH 7.5 and 25 °C.

For intracellular enzyme activity assay, the cells were disrupted by adapting the procedure reported by Dalmau et al. [11]. After harvesting by centrifugation (Hettich Rotina 35R) at 12,000 × *g* for 10 min at 4 °C, the cells were washed in Tris–HCl buffer (10 mM, pH 8.0) and resuspended to a 3 mL final volume with the same buffer. The cell suspension was disrupted with glass beads (Biospec Mini-Beadbeater, USA) for 8 periods of 30 s. The disrupted cells were centrifuged at 12,000 × *g* at 4 °C for 10 min and the supernatant was used as the cell extract for the determination of intracellular activity. The cell viability was inspected with a microscope (Olympus CX21FS1, USA) after staining the cells with methylene blue.

Protease activity was assayed spectrophotometrically (Shimadzu 1601) using casein as substrate [12]. One unit of enzyme activity is defined as 4 nmol tyrosine released/min per mL.

Biochemical assays were carried out in duplicate. The range of duplicate values was within 5%.

2.4. Cell biomass concentration

Biomass was determined by dry weight as follows [11]: samples were filtered (0.45 μm), washed with a mixture of dioxane–propionic acid (1:1), and washed with 20 mL of distilled water. The filters were then dried at 85 °C to constant weight.

3. Results and discussion

C. rugosa fermentation was carried out employing four different oxygen transfer strategies to investigate the effect of DO concentration (% saturation) adjustment and control throughout the fermentation on lipase and esterase enzyme activities.

Oxygen transfer strategies employed in the study are depicted in Fig. 1. *OTS I*. The fermentation was conducted at the level of full oxygen saturation; no decrease in DO concentration was allowed by continuous feeding of pure oxygen into the medium. *OTS II*. The fermentation was started with full oxygen saturation, a decrease was allowed in DO concentration until 60% saturation, and then this concentration was kept constant by feeding a mixture of oxygen and air gases. *OTS III*. The fermentation was started with full oxygen saturation, the level was gradually decreased to 80, 60, 40 and 30% saturations at 2-h intervals, and thereafter, 30% saturation was maintained by feeding a mixture of oxygen and air gases. *OTS IV*. The fermentation was started with full oxygen saturation, a decrease was allowed in DO concentration until 30% saturation, and then this concentration was kept constant.

The variations in pH with fermentation time for different OTSs are also depicted in Fig. 1. There were no considerable changes in pH values throughout the fermentations except for slight decreases in early periods.

3.1. Biomass

Fig. 2 shows the variations in cell biomass concentration with fermentation time for different OTSs. The accumulation rate of

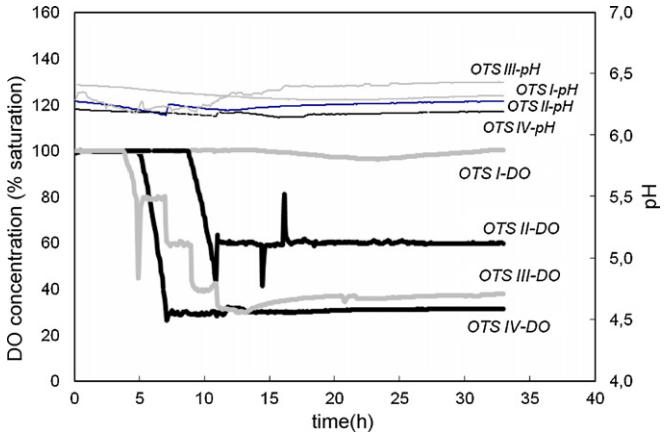

Fig. 1. The variations in DO concentration (% saturation) and pH with time in the fermentation of *C. rugosa* for different OTSs ($T = 30$ °C, $N = 500$ rpm, Q/V = 0.6 vvm, carbon source: 2 g/L triolein, nitrogen source: 4 g/L urea).